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Parallel evaluation of antimicrobial peptides derived from the synthetic PAF26 and the human LL37

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Abstract

The antimicrobial hexapeptide PAF26 was de novo designed towards phytopathogenic fungi of agricultural importance. To analyze its clinical potential, the activity of PAF26 has been determined against several microorganisms of clinical relevance including *Staphylococcus*, *Candida*, and several dermatophytes. For comparison purposes, the peptides KR20 and KI26 derived from the human cathelicidin LL37 were selected and fungal pathogens of agronomic relevance were included. PAF26 has similar antimicrobial activity in vitro compared to KR20 despite their different lengths and amino acid compositions. Moreover, neither peptide is lytic to human erythrocytes or keratinocytes. The hybrid peptide PAF26:KR20 showed better antimicrobial properties than the original peptides against most of the pathogens tested. The structural properties of PAF26:KR20 compared to related 26-amino acid peptides support the idea that the increment in toxicity correlates with positive charge and hydrophobicity. However, the degree of peptide helicity was not a predictor of antimicrobial activity.

Keywords: Cathelicidin derivates; Antifungal peptides; Antibacterial peptides

Antimicrobial peptides (AMPs) have broad-spectrum activity against bacteria, yeast, fungi, and virus, and play important roles in the defense system of many organisms from insects to humans [1]. AMPs have a low risk of resistance emergence and are being extensively proposed as new antimicrobial agents. One potential application of these peptides is the prevention and treatment of bacterial and fungal skin infections caused, for example, by Staphylococcal species and dermatophyte fungi. Particularly, mammalian skin and other epithelial surfaces are the major producers of functional cathelicidins [2,3]. The location and regulation of cathelicidin expression have indicated their importance in defence against skin pathogens [4,5]. The unique member of the cathelicidin family present in humans is hCAP18/LL37. Recently, it has been demonstrated that LL37 is produced by the eccrine apparatus and secreted into human sweat [6], where it is processed into multiple antimicrobial peptides, such as RK31 or KR20 [7].

Natural AMPs are generally cationic peptides of 13–50 amino acids and approximately 50% hydrophobic residues. However, shorter AMPs (<10 residues) have been developed by using natural AMPs as lead compounds or combinatorial approaches [8,9], to attempt to increase potency, solve protease susceptibility and tissue penetration. Previously, we identified from a peptide combinatorial library a group of antifungal hexapeptides, named PAFs, that inhibit in vivo infection of selected phytopathogens [10]. A complete study on the mode of action of one of these hexapeptides, PAF26, showed multiple detrimental effects on target fungi but not hemolytic activity [11]. Moreover, PAF26 shares amino acid sequence properties of the

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so-called cell penetrating peptides, which are translocated inside cells by an endocytosis-independent process [12]. PAF26 has been demonstrated as a short de novo designed penetratin-type peptide that is internalized into target fungal cells at sub-inhibitory concentrations [11]. Therefore, PAF26 could be a good candidate to use in clinical, beyond its initial intended agricultural application.

To compare the antimicrobial properties of peptides from distinct origins, the activity of the hexapeptide PAF26 was tested against pathogens of clinical relevance including *Staphylococcus*, *Candida*, and several dermatophytes, and also bacterial mutants widely used in the characterization of AMP activity. Likewise, we selected the cathelicidin-derived peptide KR20 and included in our study fungi of agricultural relevance (i.e. strains of *Penicillium*) for comparison purposes. KR20 has been selected for this study as a natural cathelicidin-derived peptide produced in sweat with antimicrobial potency similar to LL37 but diminished immunostimulatory functions [13]. Second, a hybrid peptide between PAF26 and KR20 and the corresponding controls were designed to characterize its antimicrobial and structural properties.

Materials and methods

Synthesis of peptides. Synthetic peptides (Table 1) were purchased from GenScript Corporation (Piscataway, NJ) with 90% purity. Peptide grand average hydropathicity index (GRAVY) [14] was calculated using a web-based tool (http://www.expasy.org/tools/protparam.html).

Microorganisms. The microorganisms used are fungal isolates of clinical and agronomic relevance (i.e. dermatophytes and strains of *Penicillium*, respectively) as well as yeast and bacterial strains (Table 2). Fungi were cultured on potato dextrose agar (PDA, Difco, Detroit, USA) for 10–14 days at 24 °C and 10× stock solutions at 2.5×10^5 conidia/mL were prepared in 1 mM sodium phosphate buffer, pH 7 (NaPB), and used in the antifungal assays. Bacteria were grown overnight at 37 °C with vigorous shaking (group B *Streptococcus* was grown without shaking) in tryptic soy broth (TSB, Sigma, St. Louis, MO, USA) and 10× suspensions at 2.5–5×10^6 colony forming units (CFU)/mL were prepared in 1 mM NaPB. Yeast were grown in a modified Dixon medium (mDixon) (4% malt extract, 0.6% Bacto Peptone, 1% glucose, and 1% Tween-80) at 37 °C with vigorous shaking for 1–2 days, as previously described [5] and 10× suspensions at 10^6 CFU/mL were prepared in 1 mM NaPB.

In vitro antimicrobial activity assays. The antimicrobial activity of the peptides was determined using a microtiter plate assay [10]. The assay mixture contained 60 μL of 1 mM NaPB, 10 μL of a 10× suspension of each microorganism, 20 μL of culture media (TSB, mDixon or PDB for bacteria, yeast or fungi, respectively), and 10 μL of a 10× stock solutions of each peptide prepared in sterile milliQ-H_2O. For fungi and yeast, the assay mixture contained 30 μg/mL chloramphenicol per well to avoid bacterial contamination. For bacteria and yeast, the 96-well microtiter plates were incubated at 37 °C and 30 °C, respectively, and the growth was determined by measuring optical density at 600 nm (OD_{600}) over time. After 1 day of incubation, aliquots of each pathogen–peptide combination were plated in agar plates and incubated at 37 °C for 1 day (3 days incubation for *Malassezia furfur*) in order to count the colonies. For filamentous fungi, the 96-well microtiter plates were incubated at 24 °C for 3 days and the growth was determined by measuring OD_{492} over time. Then aliquots of each pathogen–peptide combination were plated in agar plates and incubated at 24 °C for 3–5 days.

The antimicrobial activity of each peptide was determined using two parameters, the minimum inhibitory concentration (MIC) and the lethal concentration (LC). The MIC was defined as the lowest peptide concentration that showed at least 95% inhibition of growth. For some microorganisms, the OD measurement was impossible due to agglomeration and the MIC was determined as the lowest peptide concentration that prevented visual growth. The LC was defined as the lowest peptide concentration at which <1% of growth was recovered in peptide-free plates after treatment.

Hemolytic activity assay. The cytolitic activity of the peptides on human erythrocytes was determined as release of hemoglobin monitored by absorbance at 415 nm [11]. Peptides were used at final concentrations of 30, 50, 75, and 100 μM. Zero percent and 100% hemolysis controls were determined in PBS and 0.2% Triton X-100, respectively.

Measurement of mammalian cell toxicity. Normal human keratinocytes were cultured in EpiLife medium (Serum-Free Keratinocyte Medium, Cascade Biologics, Portland, OR, USA) supplemented with 0.06 mM calcium, 1% EpiLife Defined Growth supplement, and 1% penicillin/streptomycin. Keratinocytes cultured to confluence were incubated for 6 h at 37 °C with 100 μl of EpiLife medium containing 1, 5, 10 or 20 μM of each peptide. 100% toxicity was determined in 0.2% Triton X-100. After removal of supernatants, cells were incubated briefly with 1.3 μg/ml propidium iodide (PI) and fluorescence intensity was measured at an excitation of 530 nm and an emission of 620 nm.

Circular dichroism (CD) measurements. The CD spectra of the peptides were recorded in the far-UV (i.e. 190–250 nm) using a Jasco-810 spectrophotometer with a quartz cell of 0.1 cm path length. Peptide concentration was 30 μM in H_2O or in presence of 2 mM sodium dodecyl sulphate (SDS) or 20 mM SDS. CD spectra were measured at 25 °C with 0.1 nm step resolution, 50 nm/min speed, 1 s response time, and 1 nm bandwidth, and were averaged over 12 scans. The spectra are expressed as molecular ellipticity $[\theta]$ (deg cm^2 $dmol^{-1}$) vs. wavelength (nm) and the percentage of α-helical conformation was estimated using the software Jasco Secondary Structure Estimation.

Homology modeling. The structure of human LL37 fragment [15] was obtained from the PDB. The pair-wise alignments of this sequence with those of PAF26:KR20 and P20:KR20 were the basis of creating homology models, using the PDB entry 2FCG as the template in the program SOD [16]. The model was modified in the graphics program O [17], using rotamers that would improve packing of the protein and structural comparisons were carried out.

Table 1
Amino acids sequences and characteristics of peptides

Peptide	Sequence	MW	Net charge[a]	GRAVY[b]	Source
PAF26	RKKWFW	950.1	3+	−1.883	[10]
P20	RKTPFW	833.9	2+	−1.467	[10]
KR20	KRIVQRIKDFLRNLVPRTES	2468.9	4+	−0.755	[7]
PAF26:KR20	RKKWFWKRIVQRIKDFLRNLVPRTES	3401.0	7+	−1.015	This study
P20:KR20	RKTPFWKRIVQRIKDFLRNLVPRTES	3284.9	6+	−0.919	This study
KI26	KIGKEFKRIVQRIKDFLRNLVPRTES	3171.7	5+	−0.750	This study

[a] Estimated at pH 7.
[b] Peptide GRAVY index.

Table 2
MIC and LC data against different microorganisms

Medium	Microorganism	Peptides (μM)									
		PAF26		P20		KR20		PAF26:KR20		P20:KR20	
		MIC	LC	MIC	LC	MIC	LC	MIC	LC	MIC	LC
20% TSB	*Staphylococcus aureus* ATCC25923	30	75	>75	>75	50	>75	30	30	30	50
	Staphylococcus aureus Δ*mprF*	<5	<5	>75	>75	<5	5	<5	<5	<5	<5
	Staphylococcus aureus Δ*dlt*	<5	<5			<5	10	<5	<5	<5	<5
	Escherichia coli O29	20	20	>75	>75	5	10	10	10	10	20
	Group B *Streptococcus* A909 Ia	10	10	75	>75	10	10	<5	<5	<5	<5
20% Dixon	*Candida albicans* ATCC14053	30	30	>75	>75	20	30	20	20	20	20
	Malassezia furfur ATCC46267	20	30	>75	>75	20	20	20	20	20	20
20% PDB	*Aspergillus niger* clinical isolate	20	20	75	75	20	20	20	20	20	20
	Nannizzia otae CECT2797	30	>75	>75	>75	20	>75	10	>75	10	>75
	Trichophyton mentagrophytes CECT2958	20	20	75	>75	20	20	5	5	10	10
	Trichophyton rubrum clinical isolate	20	20	75	75	50	50	10	10	10	20
	Trichophyton rubrum CECT2794	30	50	>75	>75	20	30	10	10	20	20
	Penicillium digitatum PHI-26	10	30	75	>75	10	30	5	30	10	30
	Penicillium italicum PHI-1	10	50	50	>75	5	> 75	5	20	5	20
	Penicillium expansum PHI-65	30	50	>75	>75	20	20	20	20	20	20
	Penicillium brevicompactum PHI-8	50	75	>75	>75	30	50	>75	>75	>75	>75

Results and discussion

Antimicrobial activity of PAF26 against pathogens of clinical relevance

Previously, the hexapeptide PAF26 was shown to be antimicrobial against different phytopathogenic fungi. In this work, its spectrum of action was studied against pathogens of clinical relevance. PAF26 inhibits the growth of the gram-negative enteroinvasive bacteria *Escherichia coli* O29, gram-positive bacteria (*Staphylococcus aureus* and Group B *Streptococcus*), yeast (*Candida albicans* and *M. furfur*) and fungi (*Aspergillus niger* and several dermatophytes as *Trichophyton rubrum* and *Trichophyton mentagrophytes*) with MIC of 10–30 μM depending of the microorganism (Table 2). These values are consistent with reported MIC for other hexapeptides [8,9] and with the data previously reported for phytopathogenic fungi [18] (see Table 2 for comparison). The effect of PAF26 was lethal, killing the microorganisms with LC values close to MIC for most of the pathogens tested (Table 2). Moreover, the antimicrobial activity of the hexapeptide was sequence-specific since the sequence-related peptide P20 (Table 1) has negligible activity against all the microorganisms tested (Table 2).

Comparison of activity of PAF26 and the natural antimicrobial peptide KR20

For comparison with PAF26, we selected the cathelicidin-derived peptide KR20 previously identified in sweat (Table 1) [7], and we included selected fungal isolates of agricultural relevance (i.e. strains of *Penicillium*). Both peptides showed similar activity against the yeast *C. albicans* (Fig. 1A) and *M. furfur* and the filamentous fungi *A. niger*,

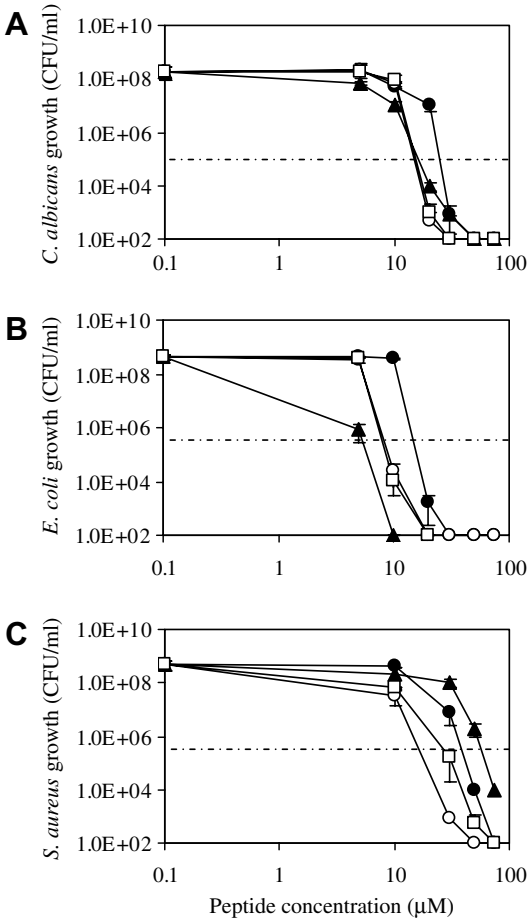

Fig. 1. Dose–response curves of antimicrobial activity of PAF26 (black circle), KR20 (black triangle), PAF26:KR20 (white circle), and P20:KR20 (white square) on *C. albicans* ATCC14053 (A), *E. coli* O29 (B), and *S. aureus* ATCC25923 (C). Initial concentration of each microorganism is indicated by a horizontal line.

natural isolates of *Penicillium* and several dermatophytes (Table 2). However, PAF26 activity against *E. coli* O29 was lower than that of KR20 (Fig. 1B and Table 2). It was initially reported that PAF26 had high activity against the fungus *Penicillium digitatum* but low toxicity against the laboratory strain *E. coli* DH5α [10].

On the contrary, PAF26 was slightly more toxic against the gram-positive bacteria *S. aureus* than KR20 (Fig. 1C). PAF26 activity was markedly increased against *S. aureus* Δ*mprF* [19] and Δ*dlt* [20] mutants (Table 2). Both mutations lead to cell envelope modifications resulting in an increase in surface anionic charge and greater binding by cationic host defence molecules [19,20]. Thus, these mutant strains amplify sensitivity to cationic antimicrobial peptides whose primary interaction with microbes is electrostatic.

For a possible clinical application, a high therapeutic index—indicating high antimicrobial but low cytotoxic activity—is essential. The selectivity of both peptides for microbial cells over mammalian cells was evaluated by assessing the degree of erythrocyte and keratinocyte lysis. As it has been previously reported [11], the hexapeptide PAF26 showed a markedly reduced cytotoxicity towards erythrocytes (i.e. 0.9% of hemolysis was obtained with 100 μM PAF26). Likewise, the 20-mer KR20 showed 0.4% hemolysis at 100 μM under our assay conditions. For comparison purposes, the cytotoxic peptide Melittin was 100% hemolytic at 100 μM under our experimental conditions [11]. In this study, we also compared the effect of these peptides on the membrane permeability of human keratinocytes by measurement of permeation to PI. Minimal membrane permeability was observed for both peptides at all concentrations tested (i.e. 5.6% and 14.5% of PI incorporation was obtained with 10 μM PAF26 and KR20, respectively, compared with the published data of 100% of PI incorporation with 10 μM of the natural LL37 [13]).

Antimicrobial activity of hybrid peptides PAF:KR20

A hybrid peptide of PAF26 and KR20 was designed to characterize and compare its antimicrobial and structural properties with the parent peptides. We designed the addition of the PAF26 sequence at the N-terminus of KR20 (PAF26:KR20) because more helical content was predicted for this combination. Previous published data correlated the helical content of LL37 with antibacterial activity [21]. A modest increase of antimicrobial activity of PAF26:KR20 compared to the original peptides was observed (Table 2 and Fig. 1). This increase was more significant against several of the filamentous fungi tested (Table 2 and examples in Fig. 2A for *T. mentagrophytes* CECT2958 or in 2B for *Penicillium italicum* PHI-1 at 20 μM peptide).

To determine if the higher activity of PAF26:KR20 is a specific effect of the PAF26 addition to KR20, we designed and tested the peptide P20:KR20 as a hybrid peptide

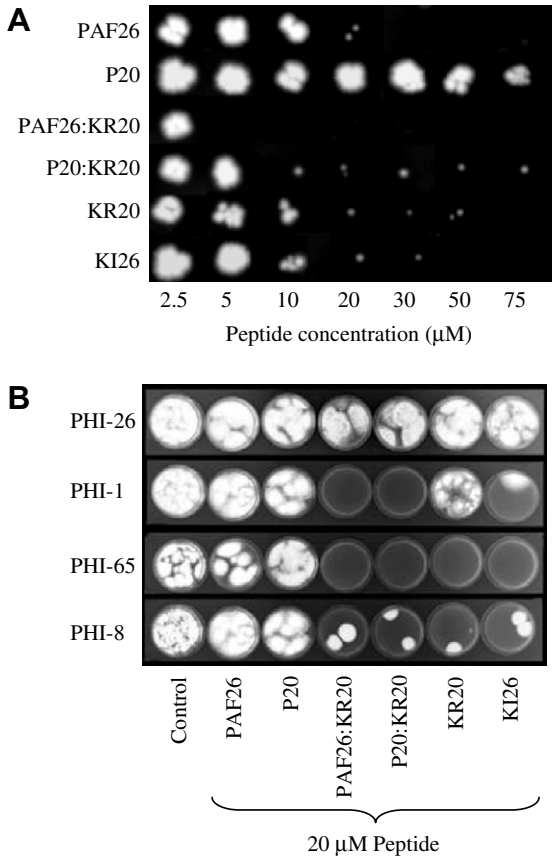

Fig. 2. Assessment of microbicidal activity of peptides against the filamentous fungi *T. mentagrophytes* CECT2958 (A) and different *Penicillium* species (B) (see Table 2). Conidia were treated with indicated peptide concentrations for 3 days and applied as drops (A) or spread (B) onto peptides-free plates.

between the inactive hexapeptide P20 and KR20 (Table 1). The peptide P20:KR20 showed similar inhibitory and killing profile as PAF26:KR20 against almost all the microorganisms tested, with modest differences against the bacteria *E. coli* and *S. aureus* and the filamentous fungi *P. digitatum* PHI-26 and *Trichophyton* spp. (Table 2). Noteworthy P20 had much lower activity than PAF26, as previously described [10]. In general, both PAF26:KR20 and P20:KR20 appeared to be more lethal than the 20-mer KR20 or the 6-mer PAF26, suggesting a possible effect of the peptide length. An additional 26-amino acid peptide which is an N-terminal extension of KR20 derived from the cathelicidin LL37 (Table 1, KI26) was therefore tested. The activity of KI26 was similar to KR20 for all microorganisms tested (Fig. 2 and data not shown), thus suggesting that the higher killing activity of PAF26:KR20 and P20:KR20 is not simply a consequence of peptide length. Comparing the antimicrobial activity of the three 26-amino acid peptides against all the microorganisms tested, PAF26:KR20 has the highest activity, followed by P20:KR20 and lastly KI26, with a correlation of antimicrobial properties with positive charge and GRAVY index (Table 1).

Structural analysis

The conformational behavior of the peptides was analyzed by means of CD spectroscopy. PAF26 adopts a random conformation in aqueous solution and an incipient α-helical conformation in 2 mM SDS [22]. The peptides KR20 and KI26 adopted a random conformation in aqueous solution at pH 7.0 (Fig. 3A and B), while P20:KR20 (Fig. 3C) has some structural ordering that the prediction software did not relate to a defined conformation. On the contrary, the spectrum of PAF26:KR20 was characterized by two negative peaks at 206 and 223 nm that might be associated with an α-helical conformation (61% prediction) (Fig. 3D). The addition of the membrane mimetic SDS at concentrations below and above the critical micellar concentration, 2 mM and 20 mM, respectively, induced formation of α-helix structures to distinct extents, depending on the peptides, and the spectra obtained showed the presence of two negative peaks at 208 and 222 nm (Fig. 3). The α-helical content with 2 mM SDS was estimated to be 52% for KR20, 45% for KI26, and 100% for P20:KR20 and PAF26:KR20 in correlation with the higher ellipticity of the hybrid peptides (Fig. 3).

The human cathelicidin LL37 was included in our analyses showing a similar behavior to KR20 and KI26 (CD spectra not shown). According to previous studies [15,21], LL37 has random conformation in aqueous solution, but adopts partly helical secondary structure upon exposure to 2 mM and 20 mM SDS (59% and 47% of α-helical content, respectively). Recently, it has been reported that LL37 shows a well-defined helical structure in the region corresponding to residues 17–30, and residues 13–16 correspond to a poorly defined peptide region [15]. Besides, Phe17 of

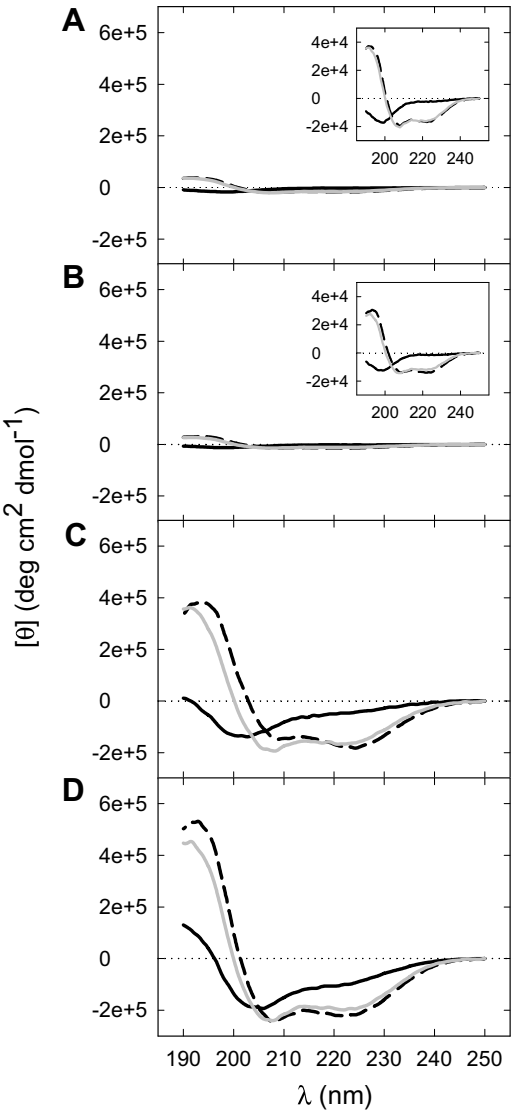

Fig. 3. CD spectra in the far-UV of KR20 (A), KI26 (B), P20:KR20 (C), and PAF26:KR20 (D) in aqueous solution (solid black) or in the presence of 2 mM (dash black) or 20 mM SDS (grey). The spectra are expressed as molecular ellipticity $[\theta]$ (deg cm^2 dmol^{-1}) vs. wavelength (nm). Insets in (A) and (B) show the same data at a different scale of ellipticity.

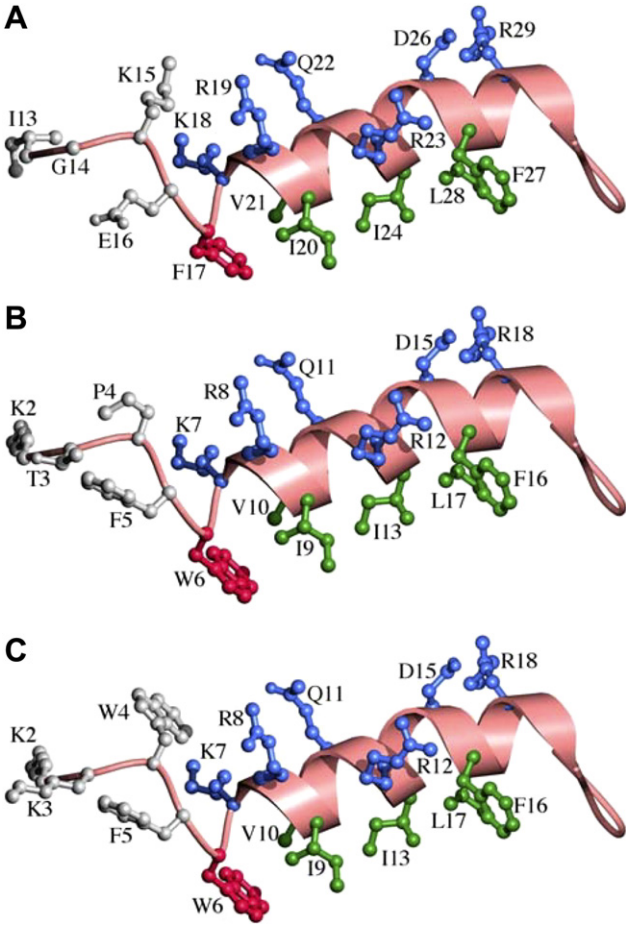

Fig. 4. Comparison of homology models of synthetic AMPs with the sequence of LL37 corresponding to KI26. (A) KI26, (B) P20:KR20, and (C) PAF26:KR20 pictures were prepared using Molscript [24] and Molray [25] showing N-terminal residues in gray, hydrophobic residues in green, and hydrophilic residues in blue. Phe17 of human LL37 and corresponding Trp residues of synthetic AMPs are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

LL37 plays an important role in the hydrophobic surface to retain the amphipathic nature, which is essential for its activity, and might form hydrophobic interactions with Val21 in LL37 [15]. Homology modeling of the synthetic antimicrobial peptides PAF26:KR20 and P20:KR20 compared with the sequence fragment of LL37 corresponding to KI26 was carried out (Fig. 4). Phe17 of LL37 is replaced with the lesser hydrophobic amino acid Trp both in PAF26:KR20 and P20:KR20, changing the hydrophobicity of the surface. However, at the same time, residues in the N-terminus (Phe and Trp) extend the hydrophobicity of PAF26:KR20, and in a minor extent of P20:KR20, as indicated also in GRAVY index (Table 2). Moreover, our predictive data suggest that these amino acids in the N-terminus can induce a better defined structure of the peptide. In fact, Glu16 in LL37 has changed to Phe in both synthetic AMPs, which possibly has interactions either with Thr3 or Trp6 in P20:KR20 and Lys3 or Trp6 in P26:KR20. In addition, the replacement of Gly14 of LL37 to Thr or Lys in P20:KR20 or PAF26:KR20, respectively, change the spatial arrangement of the surrounding. Therefore, our structural predictions would indicate that the hybrid peptides can fold into more stable α-helix, as clearly indicated by the CD data. Our data also show that the remarkable differences in α-helix folding capability among KR20, PAF26:KR20 and P20:KR20 do not correlate with the minor antimicrobial differences, according to previous published results for the rabbit cathelicidin CAP18 [23].

Conclusions

PAF26 has antimicrobial activity similar to the cathelicidin derived peptide KR20 against several pathogens of clinical relevance as *Candida, Malassezia*, and *Trichophyton,* in spite of their different length and amino acid composition. Conversely, the human cathelicidin-derived KR20 is active against fungal phytopathogens. Also, both peptides are not cytotoxic to human erythrocytes and keratinocytes. A hybrid peptide between PAF26 and KR20 showed an increase of activity, mainly against filamentous fungi, which likely is not related with its much higher α-helical content. Homology models and CD spectra of PAF26:KR20 and P20:KR20, compared with LL37-derived-peptide KI26, suggest that the amino acids in N-terminus induced a better definition of the structure in this region.

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